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MINIREVIEW

Microbial Dynamics in Traditional and Modern Sour Beer Production

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ABSTRACT Traditional sour beers are produced by spontaneous fermentations involving numerous yeast and bacterial species. One of the traits that separates sour beers from ales and lagers is the high concentration of organic acids such as lactic acid and acetic acid, which results in reduced pH and increased acidic taste. Several challenges complicate the production of sour beers through traditional methods. These include poor process control, lack of consistency in product quality, and lengthy fermentation times. This review summarizes the methods for traditional sour beer production with a focus on the use of lactobacilli to generate this beverage. In addition, the review describes the use of selected pure cultures of microorganisms with desirable properties in conjunction with careful application of processing steps. Together, this facilitates the production of sour beer with a higher level of process control and more rapid fermentation compared to traditional methods.

KEYWORDS microbial dynamics, *Saccharomyces cerevisiae*, sour beer, lactic acid bacteria, mixed fermentation

Beer is a malt-based alcoholic beverage consumed worldwide (1). The earliest written records of beer consumption date to 2800 BC, but historians believe beer or beer-like beverages were consumed much earlier. Billions of liters are consumed each year, making beer among the most popular beverages today. According to the German Beer Purity Law from 1516, beer should only contain water, malt, and hops. Yeast was later included on the ingredient list. This law, with some modifications, is still applied in countries such as Germany, but nonmalt carbohydrate sources are extensively used in beer production worldwide (1).

Malt, usually wheat or barley, is milled and mixed with hot water in a mashing step. During the mashing, enzymes, including α- and β-amylases, degrade starch to fermentable carbohydrates. After mashing, the insoluble fraction, referred to as brewer's spent grain (BSG), is separated from the sugar-rich liquid, referred to as wort, in a process called lautering. The wort is then boiled with hops before it is cooled and inoculated with yeast (Fig. 1A). The most commonly used yeast species for beer fermentation, also known as brewer's yeasts, are *Saccharomyces pastorianus*, used for fermentation of lager beer, and *Saccharomyces cerevisiae*, used in ale production. During fermentation, the yeast, usually a single-strain culture, utilizes the available carbohydrates, amino acids, and other nutrients in wort to generate ethanol, carbon dioxide, higher alcohols, esters, and other metabolites (1).

Different processing steps reduce the beer's susceptibility to unwanted microbial growth during production. Examples of such processing steps include malt acidification, application of high temperatures during mashing, boiling, and pasteurization in addition to filtrations and application of low temperatures during storage (2). Furthermore, hops containing antimicrobial iso-α-acids (typically 17 to 55 mg/liter) also act as preservatives. By going through the fermentation process, beer typically acquires a

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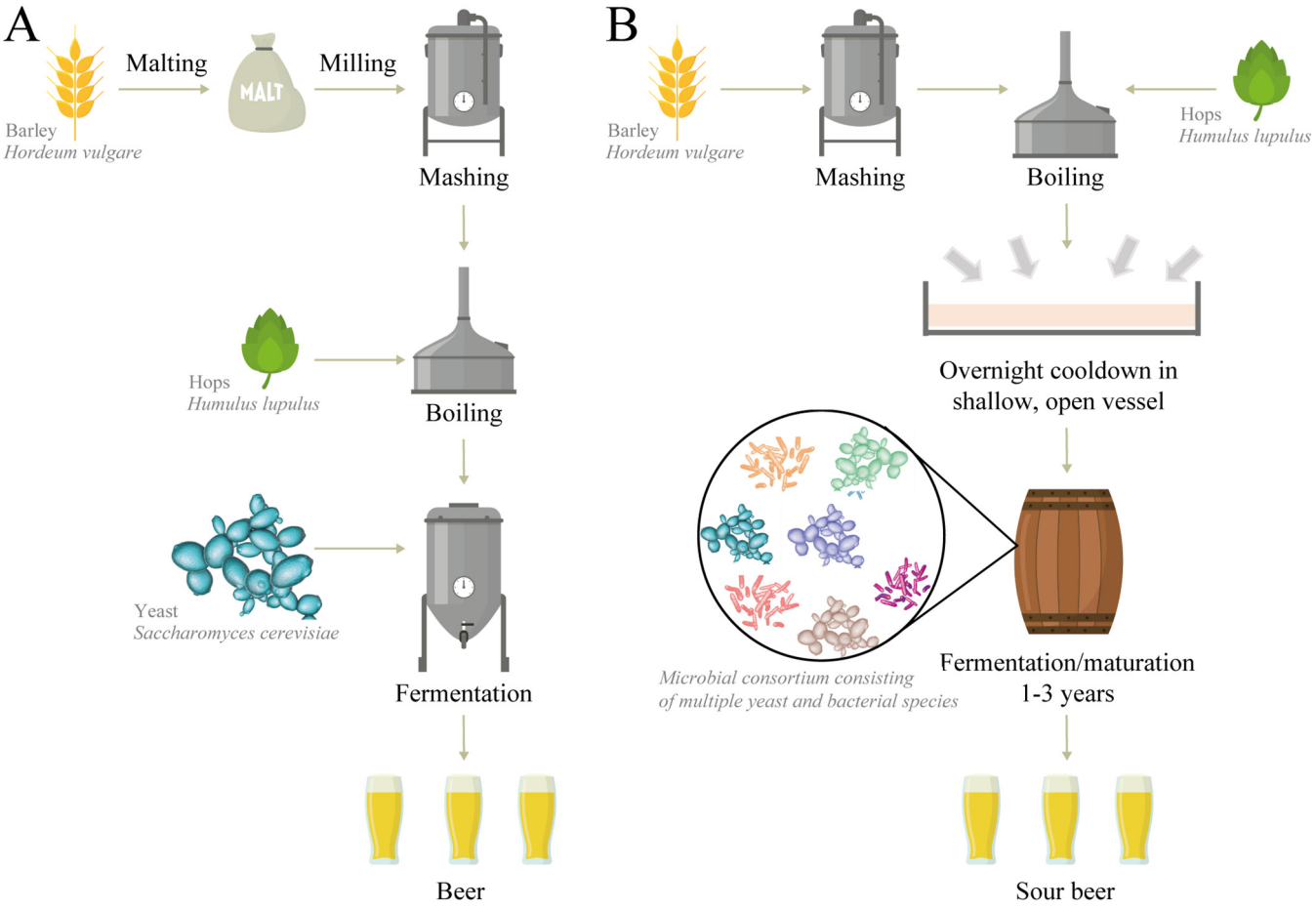

FIG 1 (A) Schematic illustration of the beer production process. Grain is malted, milled, and mashed before wort is separated from brewer's spent grain and boiled with hops. Yeast is added to chilled wort to ferment the sugary wort into ethanol-containing beer. (B) Schematic illustration of the lambic beer production process. Active inoculation of wort is not carried out. Boiled wort is cooled down in a shallow, open vessel (coolship), where it is spontaneously inoculated by exposure to the environment. The wort is transferred to wooden casks, where spontaneous fermentation by a variety of yeasts and bacteria can transpire.

number of properties that make it an inhospitable environment for microbial growth, thus protecting against spoilage (2). These factors include ethanol, typically in the range of 3.5% to 5% or higher, acidic pH, low oxygen, and high carbon dioxide content as well as low quantities of available nutrients.

Ethanol in beer provides an important antimicrobial hurdle. In 1935, Shimwell showed that beers with higher ethanol content were more resistant to growth of *Lactobacillus brevis*, which was referred to as *Saccharobacillus pastorianus* at that time (3). The antimicrobial mode of action of ethanol is through inhibition of cell membrane function (4) and induction of cell membrane leakage (5). An ethanol-induced increase in membrane permeability causes a rise of protons influx into the cytoplasm, which makes it difficult for bacterial cells to maintain pH homeostasis (6). This is especially important in low-pH environments such as beer. Cell morphology and a variety of cellular functions can also be affected by ethanol (7).

Low pH represents an additional hurdle that microorganisms need to circumvent to grow in beer. Beer pH generally ranges between 3.4 and 4.7, depending on beer style, but most beers have a pH ranging between 4.0 and 4.5 (8). Acidic pH causes increased influx of organic acid and acidification of the cytoplasm. This can damage various enzyme systems and hinder nutrient uptake, thereby interrupting cellular metabolism in general (9). Inability to maintain constant intracellular pH results in cell death (10). In addition to the direct effect of low pH, the acidic environment affects microbial cells survival synergistically with hop compounds (11).

When hops are added to beer, they introduce various antimicrobial compounds such as α-acids, iso-α-acids, and β-acids. Iso-α-acids are the most important antimicrobial compounds acting primarily as ionophores (12). Being weak acids, undissociated iso-α-acids can cross cell membranes and dissociate intracellularly where the pH is higher (13). The release of protons causes a drop of the intracellular pH that demolishes the proton motive force, ultimately affecting the whole-cell metabolism (13). Other antimicrobial actions inherent to iso-α-acids include induction of membrane leakage (14) and oxidative stress in the presence of manganese at low pH (15).

Carbon dioxide is formed during yeast fermentation of beer; CO_2 lowers beer pH and contributes to making it microbiologically stable. Further, the presence of CO_2 creates an anaerobic environment that inhibits growth of aerobic bacteria (2). CO_2 acts as a preservative through pH reduction and oxygen displacement and through an inherent antimicrobial effect that is not yet fully elucidated (16). An inhibitory effect of CO_2 on a number of metabolic enzymes has been suggested as an important mode of action (17), as has disturbance of cell membrane function (18). Regardless of the mechanism, CO_2 exposure inhibits growth in both Gram-positive and Gram-negative bacteria (19), and higher levels of CO_2 in beer have been associated with reduced growth of beer spoilers (20).

During fermentation, yeast will consume the majority of nutrients. The available quantities of carbohydrates and amino acids in most beers are therefore low (21). Low nutrient content has been correlated with decreased susceptibility to bacterial growth (22).

Although the hurdles described above make the beer stable with respect to microbial growth, there are microorganisms capable of contaminating beer. The presence of microorganisms with beer spoilage potential can cause loss of colloidal stability, ropiness, and aroma and taste defects, among others (23). Lactic acid bacteria (LAB) (24), acetic acid bacteria (AAB) (25), *Enterobacteriaceae* (26), *Zymomonas* and *Pectinatus* spp. (27), and *Megasphaera* spp. (28) are all bacteria associated with beer spoilage. Some yeasts, including *Brettanomyces*, *Candida*, *Hanseniaspora*, *Torulaspora*, *Pichia*, and *Saccharomyces*, also have beer spoilage potential (29). It is a common belief that beer is resistant to foodborne pathogens. Some studies have, however, suggested that some foodborne pathogens, such as strains of *Escherichia coli* and *Bacillus cereus*, are able to survive in beer (30). In the context of sour beers, microorganisms with beer spoilage potential can be viewed in a different light, as the involvement of microorganisms beyond conventional brewer's yeast is essential for the production of such beers.

Sour beer. Sour beer is a highly diverse genre of beer, not restricted to one specific definition based on production process, raw material, or geographic origin. A common denominator for sour beer is higher concentrations of organic acids, causing reduced pH (pH 3.0 to 3.9) compared to "regular beers." This leads to elevated intensity of corresponding sensory attributes such as acidic taste (31). The elevated levels of organic acids in sour beer originate from the involvement of acid-producing bacteria in the fermentation process. While the fermentation of conventional beer is usually limited to single-strain yeast fermentations, sour beer originates through fermentation by multiple microorganisms, including both yeasts and bacteria (32). Various techniques for sour beer fermentations exist, including spontaneous fermentation, controlled mixed fermentations, and sour mashing and similar techniques, where the different microorganisms are separated in time (33). Belgian brewing culture is famous for its sour beer traditions, and classic sour beer styles of Belgian origin include lambic and lambic-derived beers such as geuze and kriek, as well as Flanders red ale and old brown ale. Berliner Weisse and Gose are sour beer styles of German origin (33). American coolship ale (ACA) is a product from the American craft beer culture, with a production process heavily inspired by the classic Belgian styles (34). The popularity of sour beer has increased in recent decades, and research is being carried out on both traditional fermentations and alternative production techniques. The main focus of the

current review, besides traditional sour beer products and challenges associated with their production, is on lactic acid bacteria, their adaption strategies to beer environments, and their application in modern fermentation methods. Other microorganisms, such as *Brettanomyces* and acetic acid bacteria (AAB), are also important in sour beer fermentations; their role in sour beer production has been extensively reviewed in recent publications (33, 35–38) and will not be covered in detail here.

Brettanomyces (also known as *Dekkera*) *bruxellensis* is the species most commonly associated with beer fermentations and is the cause of "Brett character," which includes fruity, floral, and tropical taints, as well as medical, leathery, smoky, and horsey aromas (39). Interest in *Brettanomyces* within the brewing industry is due to its ability to generate a wide range of flavor-active compounds, including volatile phenolic compounds (40) and volatile esters (41, 42). Further, the β-glucosidase enzymes, inherent to a number of *Brettanomyces* strains (43, 44), facilitate liberation of volatile flavor compounds bound with glycoside bonds in plant materials. Examples include the release of flavor-active compounds from cherries during traditional kriek production (45).

AAB are obligately aerobic bacteria that produce acetic acid as one of their main metabolic products (46). AAB are recognized in the production of vinegar, vitamin C, and cellulose, but are often considered problematic in the beverage industry due to their spoilage potential (47). Despite this, they are vital contributors in the fermentation of a number of products, including cocoa and water kefir, and some AAB, such as *Acetobacter* and *Gluconobacter*, are also important in spontaneous fermentations of sour beers (33, 47, 48). The produced acetic acid is important to the pH and sensory acidity of sour beer, but AAB has also been associated with other compounds important to sensory perception, such as ethyl acetate (49).

Traditional sour beer products. Lambic beers are produced through spontaneous fermentations in which no active inoculation of microbial starter cultures is carried out (Fig. 1B). The boiled wort is transferred hot to shallow, open vessels, known as coolships, and left to cool down, completely open to the air, typically overnight (32). This exposure is assumed to facilitate inoculation by environmental microorganisms present in air in the brewhouse (50–52). Microbial inoculation may also occur from the barrels, which potentially host a large number of microorganisms in a dormant stage in microcavities on the wood surface (53). To ensure that the cooldown occurs within a reasonable amount of time, and as a means for some level of microbial control, traditional lambic brewing is only carried out during the winter months (32, 51). When it reaches the temperature of approximately 20°C, the wort is transferred to wooden barrels for fermentation and maturation (32). According to the studies carried out with classic culture-dependent techniques, a four-phase microbial succession takes place during fermentation in the wooden barrels. The first phase is often referred to as the enterobacteria phase, as enterobacteria are dominating. Acetic acid bacteria and oxidative yeasts are also present during this phase, which can prevail for a week (52) to a month (32, 55). Low concentrations of ethanol and organic acids are produced during this first phase (52). The following phase is the main fermentation phase, in which *Saccharomyces* spp. dominate for 3 to 4 months, followed by an acidification phase dominated by LAB and AAB. Production of ethanol and carbon dioxide dominates the main fermentation phase, and organic acids such as lactic acid and acetic acid are produced during the acidification phase (52). The final phase is the maturation phase, where *Brettanomyces* as well as *Lactobacillus*, *Pediococcus*, and acetic acid bacteria dominate, usually from approximately 8 months onward (32). Production of esters such as ethyl acetate and ethyl lactate are characteristic of the maturation phase (51, 52). More recent studies have been carried out using culturing methods in conjunction with high-throughput sequencing techniques to obtain higher-quality information on the microbial species diversity. Spitaels et al. (55) showed that samples acquired throughout the fermentation process from two batches from a lambic brewery had a similar microbial succession to that reported by Van Oevelen et al. (32), with an initial *Enterobacteriaceae* phase the first month, followed by a phase dominated by *Saccha-*

romyces spp. and *Pediococcus damnosus*, until *Dekkera bruxellensis* dominated after 6 months. This study, however, suggested that acidification and alcohol fermentation occurred simultaneously, rather than as an extended acidification phase as described previously (32, 51). These results corresponded well with those of Bokulich et al. (34), where samples obtained during a 3-year fermentation period of spontaneously fermented American coolship ale were analyzed. Another study on lambic beer has resulted in more than 2,000 microbial isolates throughout the 2-year fermentation, of which 400 were bacterial strains and more than 1,700 were yeast strains (52). The authors describe a distinct four-phase microbial succession, with an enterobacteria phase (first week), a main fermentation (24 h to 7 weeks), acidification (week 7 to 9 months), and maturation (6 months and onward). While the enterobacterial phase lasted for a month in traditional lambic production without wort acidification (55), De Roos et al. (52) showed that the enterobacterial phase did not occur when the wort was acidified by lactic acid addition. In a study focusing on Belgian red-brown acidic ales, the authors showed that the dominant operational taxonomic units (OTUs) are *Pediococcus*, *Acetobacteraceae*, *Lactobacillus*, *Dekkera*, and *Pichia*. Lactic acid and ethanol were the main metabolites, and ethyl acetate, isoamyl acetate, ethyl hexanoate, and ethyl octanoate were identified as the main aromatic compounds (56).

Some industrial breweries produce lambic beers on a larger scale in a process that diverges somewhat from the traditional one. These breweries usually use modern processing methods, such as pasteurization, filtration, and forced carbonation for their lambic products (57). By using modern equipment to chill wort, the production can be carried out year-round, not depending on low winter temperatures for overnight cooling in shallow vessels. Industrial lambic breweries also use wooden casks, but these are generally custom-made and far greater in size (170 to 200 hl) compared to the retired wine casks used in traditional lambic breweries (57). Comparison of the microbial succession during a 1-year fermentation in an industrial lambic brewery and that occurring during traditional production identified a core microbiota (57). Microorganisms in this community included *S. cerevisiae*, *S. pastorianus*, *D. bruxellensis*, and *P. damnosus*. Differences between traditional and industrial fermentations included an absence of the *Enterobacteriaceae* phase, explained by reduced initial pH due to lactic acid addition and a larger variety of AAB in industrial production.

The microbiota living on the inner surface of the wooden casks used in a traditional lambic brewery has been shown to vary with barrel cleaning procedures and the general condition of the casks with respect to age, wood thickness, and wood porosity. Based on 16S rRNA gene sequencing, De Roos et al. (53) identified a variety of bacteria, including *Pediococcus*, *Lactobacillus*, and *Acetobacter*, and yeasts such as *Saccharomyces*, *Dekkera*, and *Pichia* possibly acting as a source for microbial inoculation (45).

Lambic is the base beer for a variety of different beer styles. Geuze (also referred to as gueuze) is a highly carbonated beer that is made by mixing young 1-year-old and older (2 years or more) lambic following refermentation in bottles. Faro is made by mixing lambic with rock sugar (58). Kriek is a fruit lambic made by mixing sour cherries with a young lambic, allowing a second fermentation on the fruit sugars (59). Raspberries can also be used in the same way in lambic beer, resulting in a product referred to as framboise (58).

For ACA production, wort is cooled in open, shallow cooling vessels to favor spontaneous inoculation by the environment before transfer to wooden barrels. The microbial succession is similar to that of Belgian lambic, although some minor differences can be found (34).

Flanders red ale and old brown ales are originally products of spontaneous fermentation and year-long maturation. Besides traditional methods, modern production of these beers is carried out as controlled mixed fermentations in which inoculated yeast and bacteria ferment the wort before young beer is matured (60). Flanders red ale originates from West Flanders, is red colored, and is said to be "wine-like." Flanders red ale is matured for up to 2 years in oak barrels. Maturation in oak separates Flanders red

ale from the old brown ales indigenous to eastern Flanders. The latter are described as more malt driven and less acidic (61).

Gose and Berliner Weisse are common German sour beer styles in which wheat malt makes up a substantial fraction of the malt bill, and lactobacilli play important roles in fermentation. Both beer styles, originating from Goslar and Berlin, respectively, represent products that are produced both through traditional and with more modern methods. An important difference between Berliner Weisse and Gose is the spiciness of the latter, imposed by addition of salt and coriander (33).

Challenges associated with traditional sour beer production. Production of sour beer through spontaneous fermentation is associated with several challenges. These include inconsistent product quality, wastage due to failed fermentations, and time consumption. A study by Spitaels et al. (62) looking at microbiota and metabolites of aged geuze clearly demonstrated inconsistency in production, as the bottle-to-bottle metabolite variation made it impossible to generalize age effects on geuze. The product variations that arise through the traditional process can be seen as a positive attribute and are greatly appreciated by some consumers, as they represent a mark of authenticity and natural production. The product inconsistency can also be considered negative, especially if beer has to be discarded due to organoleptic failure after years of fermentation and maturation. The idea of using pure cultures in controlled mixed fermentations is appealing not only because it can offer improved process control and product consistency and potentially reduce production time for sour beers, but also because controlled mixed fermentations offer tremendous potential for generation of novel products. Mixed fermentations of beer with pure cultures are utilized to an increasing extent in the craft brewing industry. The application of mixed cultures and nonconventional microbial strains to beer fermentation offers vast possibilities for flavor generation. In addition, the capacity of nonconventional brewing microorganisms for diverse carbohydrate utilization allows the inclusion of nonconventional raw materials in beer production. This can be exploited as a tool to improve process control, besides being a method for direct conversion of nonfood carbohydrate sources to food products through fermentation. *Lactobacillus* sp. are highly interesting in this regard. An example of this was recently presented, using xylooligosaccharides for controlled fermentation with *Lactobacillus* (63). Interestingly, this study revealed an interesting ratio of acetic acid to lactic acid that may favorably prevent extensive *Acetobacter* fermentation that is considered challenging in many products.

Lactobacilli and sour beer. Lactobacilli are Gram-positive rod-shaped bacteria that produce lactic acid as the main metabolic product of carbohydrate metabolism (64). Their metabolism is classified either as obligately homofermentative, meaning that they convert hexose sugars to lactic acid almost exclusively, or as obligately or facultatively heterofermentative, converting hexose sugars to lactic acid as well as CO_2 and ethanol or acetic acid. Lactobacilli have a great safety record, and certain strains of some species are used as health-promoting probiotics as well as starter cultures for fermentation of a vast variety of food products. They are associated with fermented dairy products such as yogurts (65) and cheeses (66), fermented vegetables (67), and fermented meat products (68, 69). Lactobacilli are also vital contributors to the production of a number of food products through mixed fermentations, where both bacteria and yeast participate, including kefir (70), water kefir (71), sourdough bread (72), and alcoholic beverages such as wine (73), sake (74), and beer (2).

In beer, lactobacilli can be terrible spoilers or vital fermentation contributors, depending on the beer style and the strain properties. Lactobacilli are considered spoilers in ales and lagers, as these bacteria cause unwanted haze and sedimentation, off-flavors, acid formation, and ropiness (75). In sour beer, where production of acid is welcomed, lactobacilli can be appreciated contributors, vital to the wanted organoleptic characteristics developed through fermentation. Regardless of their presence as spoilers or as needed fermenters in beer, lactobacilli need to overcome the compre-

hensive sum of hurdles to be involved. A wide set of systems for detection and adaptation to stress are involved in this (21, 76).

Lactobacilli are generally inhibited from growing in beer by the presence of hop's iso-α-acids. Some strains, however, are resistant to the antimicrobial actions of hops and thus able to survive in beer (2, 75, 77). Genes associated with hops resistance in LAB include *horA*, *horC*, and *hitA* (78). The *horA* gene encodes an ABC transporter capable of expelling hops bitter acids from cells (79). The *horC* gene presumably encodes a proton motive force (PMF)-dependent multidrug effluence pump (80, 81). Products from *horA* and *horC* contribute to hops resistance by lowering the net influx of hops bitter acids into the cell cytoplasm, thereby restricting their actions as antibacterial protonophores. The *hitA* gene is assumed to encode a divalent cation transporter that increases hop resistance by helping hop-sensitive bacteria transporting divalent cations, e.g., Mn^{2+}, into cells where the proton gradient has been dissipated (82). Other cellular adaptations are also involved in hop resistance in LAB, including modifications of the cell wall (83) and cell morphology (84). The presence of *horA* and/or *horC* is used as a genetic marker for the ability to survive in beer (68).

Lactobacilli are generally tolerant to ethanol, which confers them competitive advantages in fermentative environments (85). They do, however, display huge variation in their resistance, as some (e.g., strains of *Lactobacillus plantarum*) stop growing at 5% to 6% ethanol, while others can sustain environments with much higher concentrations (86, 87). While most LAB are inhibited above 13% ethanol (88), reports exist of sake spoilers able to grow at 20% ethanol (74). Kleynmans et al. (89) reported lactobacilli able to resist 16% ethanol, even at a pH as low as 3.3. Even though lactobacilli are generally able to sustain the ethanol levels in many beers, the role of ethanol tolerance on beer spoilage potential is not well characterized (90). Indeed, Pittet et al. (90) found no correlation between ethanol tolerance and ability to grow in beer.

Carbohydrate catabolism by lactobacilli causes accumulation of organic acids and a reduction in pH in the environment in which they reside, making it inhospitable for many potential microbial competitors. Extracellular, undissociated acids can pass cell membranes, where they dissociate in response to the higher intracellular pH, ultimately affecting enzyme activity and damaging DNA (91). Lactobacilli are not unaffected by acidic environments, even though they inflict such an environment upon themselves. Strategies involved in their response to acidic stress include the glutamate decarboxylase (GAD) system. In the GAD system, extracellular glutamate is internalized and decarboxylated to γ-aminobutyrate (GABA) in a reaction where a proton is consumed before the decarboxylated product is exported to the extracellular environment. This consumption of intracellular protons contributes to increased intracellular pH. In addition, the decarboxylation can be coupled to an electrogenic transporter, which allows ATP generation through the proton motive force (68, 92). The arginine deaminase pathway (ADI) (93) is another system for maintaining pH homeostasis in lactobacilli (94) and other LAB (95). In the ADI pathway, arginine is converted to ornithine, ammonia (NH_3), and carbon CO_2, and ATP is generated. NH_3 is generated in the conversion, and it reacts with intracellular protons, thus contributing to alkalization of the cytoplasm. The F_oF_1-ATPase is a ubiquitous enzyme among bacteria that can facilitate the production of ATP in a reaction sustained by transmembrane proton motive force, or it can expel protons from cells in an energy-consuming process sustained by ATP consumption (96). Active proton expulsion increases in acidic environments and is vital for maintaining pH homeostasis in lactobacilli (97) and other LAB (98). Several other systems are known to be involved in the acid stress response of LAB comprehensively, which are covered in the review by van de Guchte et al. (68).

Lactic acid bacteria are known to be more resistant toward the presence of CO_2 than many other bacteria (99). In addition, they are able to sustain low oxygen levels, as lactobacilli are anaerobic or aerotolerant (64).

During the fermentation of wort, conventional brewer's yeast utilizes sucrose, fructose, glucose, and maltose. Some strains can also utilize maltotriose. Poly- and

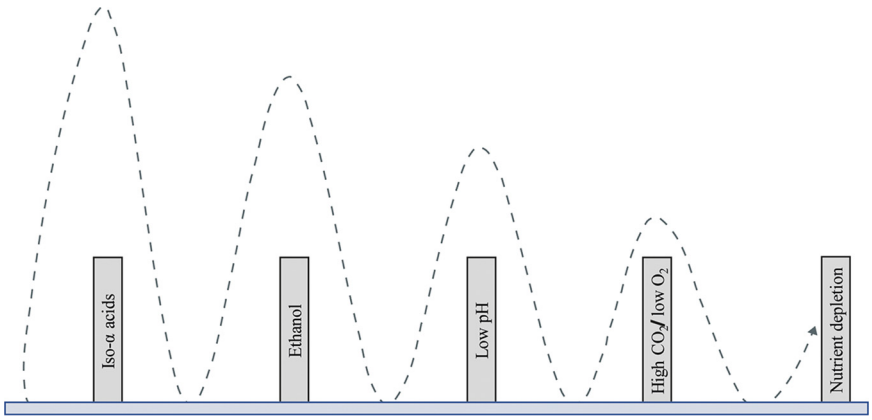

FIG 2 Illustration of the hurdle effect in beer, where relatively low-intensity hurdles such as iso-α-acids, ethanol, low pH, high CO_2, and low O_2 together pose a substantial antimicrobial effect.

oligosaccharides are also present in wort, often referred to as dextrins (100). Dextrins can contribute to the sensory perception, e.g., fullness, in ale or lager beer, but in mixed fermentations, these higher molecular mass glycans can serve as the substrate for microorganisms with carbohydrate-degrading capabilities exceeding those of conventional brewer's yeast. In traditional lambic production, a higher content of such polysaccharides is promoted by inclusion of unmalted wheat in the grain bill ($\leq$30%) and the application of turbid mashing. Both of these factors contribute to reducing enzymatic carbohydrate degradation during mashing, promoting a higher dextrin content in wort, which is assumed to be important for sustaining the prolonged fermentation phases that occur after the main fermentation in lambic production (37). Many lactobacilli have enzymes that facilitate utilization of residual carbohydrates in wort, which are not degradable by conventional brewer's yeast. Maltotriose, maltotetraose (101), maltopentaose, and more complex maltodextrins can sustain growth of *Lactobacillus* (102), and genes encoding enzymes necessary for cellular import and degradation of maltodextrins have been identified (103). Amylolytic lactobacilli can also degrade starch (104), and some lactobacilli can also utilize cellobiose (105) and xylooligosaccharides (63) (discussed in detail below). *Lactobacillus* involvement in superattenuation of lambic beer has been implicated. In superattenuated or overattenuated beer, a larger carbohydrate fraction has been fermented than the one that is degradable by brewer's yeast alone (106). Although it is not the primary focus of the current review, it should be noted that other microorganisms, including *Brettanomyces*, are able to degrade complex carbohydrates and are equally important in superattenuation of sour beer (39, 107).

As previously stated, lactobacilli must overcome the sum of hurdles in beer posed by ethanol, low pH, the presence of iso-α-acids (and other hops compounds), and nutrient depletion (Fig. 2) to carry out metabolism in the beer environment. If *Lactobacillus* growth is required, e.g., in sour beer production, this can perhaps be promoted by removing or reducing the stringency of one of the hurdles discussed above, e.g., nutrient depletion. A specific substrate, known to promote metabolism of a limited number of microorganisms, could, for instance, be added to beer to promote a rapid acidification phase in mixed or sequential fermentations. An example of such a substrate could, for instance, be lactose, which does not promote the growth of *S. cerevisiae* but supports *Lactobacillus* metabolism (108).

Modern methods of sour beer production. Producing sour beers in controlled fermentations with pure cultures is by no means a new idea. In the late seventies, a study on the microbiology of spontaneous wort fermentation suggested the following question for future research: "Can lambic be made with pure cultures?" (32). After 4 decades, there is still little evidence in the scientific literature of it having been pursued. Indeed, most of the scientific literature is focused on characterizing the microbiology

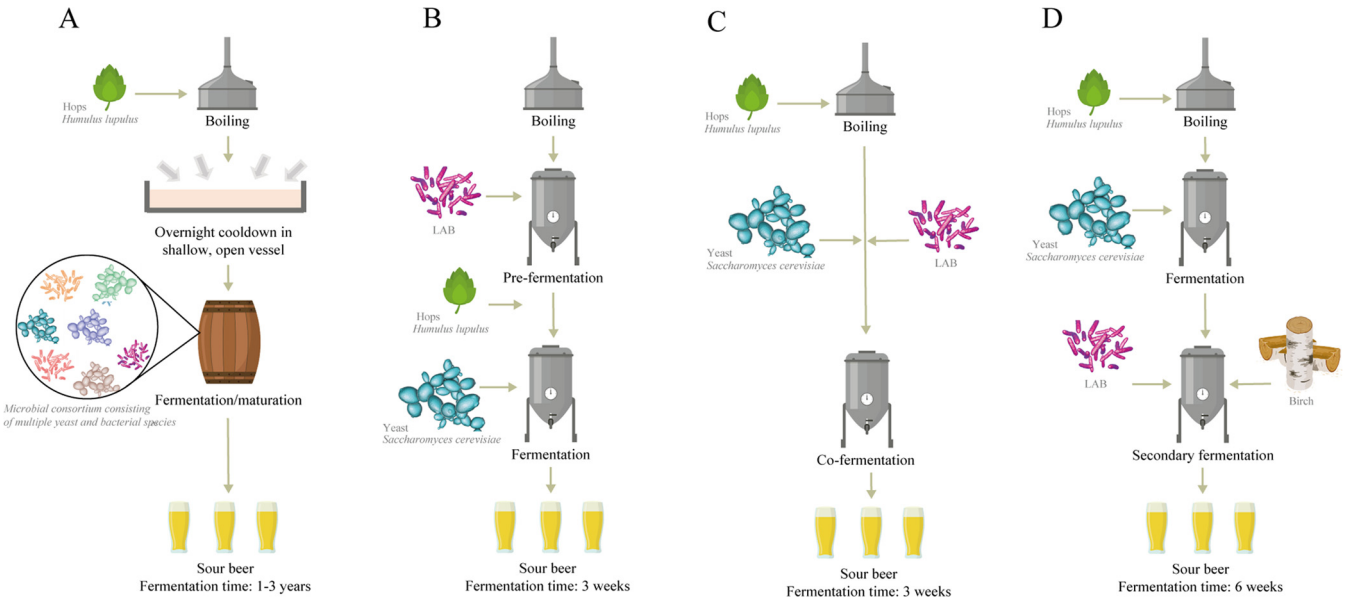

FIG 3 Modern approaches to sour beer production. (A) Traditional production process with spontaneous fermentation. (B) Prefermentation with LAB, followed by yeast fermentation. (C) Cofermentation with yeast and LAB. (D) Secondary fermentation with LAB with wood-derived carbohydrates as the substrate.

and metabolite formation of spontaneous fermentation (Fig. 3A), rather than investigating alternative production methods that may offer improved process control and/or reduce fermentation times. Experimentation into, and development of, alternative production methods have emerged in industry, and different modes of spontaneous, semispontaneous, and pure-culture fermentations are carried out for commercial production. An example of this is the "sour worting" method (Fig. 3B) where *Lactobacillus* fermentation for acid production is carried out prior to yeast fermentation, either by *Saccharomyces*, *Brettanomyces*, or both, in oak barrels (31).

A strategy for simplifying and shortening the production process was explored by Kumara and Verachtert (107). They fermented wort from a lambic brewery for a short period (≤48 h) at high temperature (28°C) with *S. cerevisiae* to obtain wort depleted of *S. cerevisiae*-fermentable carbohydrates. The yeast cells were then removed, and the prefermented wort was pasteurized before inoculation with a mixed population from spontaneously fermenting, 1-year-old lambic. In the same manner, a lambic at an earlier fermentation stage and higher carbohydrate content was pasteurized and reinoculated with the same mixed population from the further progressed lambic fermentation. Using this process, the overattenuation occurred in 30 days at 28°C, resulting in beers with more than 4,000 mg/liter lactic acid and 800 mg/liter acetic acid in both fermentations.

Single-strain fermentation with nonconventional, acid-producing yeast has also been attempted. Domizio et al. (109) tested three different strains of *Lachancea thermotolerans* in 3-week-long fermentations of wort at 14°C in which they compared the performance of *L. thermotolerans* to that of a conventional *S. cerevisiae* brewing strain. All the nonconventional strains were able to degrade maltose but not maltotriose. They were also able to produce comparable quantities of ethanol (approximately 5% vol/vol) and higher quantities of lactic acid than *S. cerevisiae*. A substantial increase in acidity was obtained with one of the tested strains (final pH, 3.77 compared to 4.24 for *S. cerevisiae*). Even though the lactic acid content was higher for all *L. thermotolerans* fermentations than *S. cerevisiae* fermentation, it only ranged from approximately 100 to 300 mg/liter, which is substantially lower than in most sour beers. Osburn et al. (110) tested 284 (54 species, 26 genera) yeasts isolated in small-scale beer fermentations for their fermentation performance. Sensory testing of the resulting beers illustrated that many of the strains generated beers described as tart or sour. The authors identified

multiple yeast strains capable of producing lactic acid and used four of these (strains of *Hanseniaspora vineae*, *Lachancea fermentati*, *Schizosaccharomyces japonicus*, and *Wickerhamomyces anomalus*) in following brewing experiments where the wort was incubated at 21.7°C for 1 month. Quantification of the lactic acid in the beers ranged from 900 to 4,500 mg/liter, and the *W. anomalus*-fermented beer was perceived as very sour, with pear, apple, and apricot aroma (110). This method, named "primary souring," is as an alternative production route for sour beer, solely relying on fermentation with yeasts that produce lactic acid as well as ethanol and CO_2.

The application of an initial biological wort acidification step (Fig. 3B) is another alternative production method for sour beer that has been explored both in industry (31) and in research (111). Biological acidification can be carried out in the mashing tun (sour mash), in the brewing kettle (kettle sour), or after the wort has been transferred to the fermentation vessel (sour wort). The concept is to carry out LAB fermentation in unhopped wort prior to yeast fermentation within a short time frame, typically 24 to 48 h. In this way, the hurdle effects imposed by yeast fermentation (ethanol, nutrient depletion, low pH, etc.) and iso-α-acids on LAB metabolism can be circumvented, and the ability of LAB to rapidly produce high quantities of lactic acid is exploited. When the desired level of lactic acid has been obtained, the wort is then boiled to stop bacterial fermentation, followed by single-strain fermentation with conventional brewer's yeast. An alternative to the interfermentation boiling step is addition of highly hopped wort upon yeast addition to introduce antimicrobial iso-α-acids after the wanted bacterial activity has transpired (112). In a study by Peyer et al. (111), *Lactobacillus amylovorus* was used for biological acidification of mash and preboil and postboil worts. Acidified worts were subsequently inoculated with *S. cerevisiae* US-05. The authors showed how biological acidification at different time points in the preyeast fermentation process led to differences in the obtained beer product. Acidification of preboil wort emerged as an efficient method to ensure high acidity and minimal organoleptic failure (112). Prefermentation with *Lactobacillus buchneri* prior to yeast fermentation was tested for production of sour beer (112). Sour beers (pH 3.5 to 3.7) with high lactic acid concentrations (~1,000 mg/liter) were produced in 3 weeks of fermentation. Although *L. buchneri* made a significant contribution to the metabolite composition of the beer, the sensory influence of this did not surpass the influence obtained with chemical acidification.

Two recent studies have explored novel strategies to expedite sour beer production and improve the process control through cofermentation of yeast and lactic acid bacteria tolerant to brewing-related stresses (113) and through secondary fermentation using a woody biomass-derived substrate (63) containing xylooligosaccharides that are also found in BSG. Two different lactobacilli, *L. plantarum* WildBrew Sour Pitch and *L. brevis* BSO464, were selected based on their ability to sustain various beer-related stress factors (ethanol, low pH, iso-α-acids, etc.) and were used in separate cofermentations with yeast (113). Sour beers (pH 3.6 to 3.8) with high lactic acid concentrations (~1,800 to 2,600 mg/liter) were successfully produced in as little as 3 weeks (Fig. 3C). *L. plantarum* contributed to the sensory properties of beer by causing increased intensity in fruity odor and dried fruit odor, while the *L. brevis*-fermented beer had similar sensory properties to a commercial sour beer in acidic taste and astringency. In another study, Dysvik et al. showed that xylooligosaccharides (XOS) from birch wood can be used to selectively support *L. brevis* BSO 464 growth in the beer (63) (Fig. 3D). Sour beer with a pH of 3.3 to 3.6 and a lactic acid concentration of 1,750 to 3,900 mg/liter was produced in only 2 to 4 weeks. XOS-driven secondary fermentation shifted multiple sensory properties significantly, and sensory evaluation of the produced XOS sour beer showed that the product was similar to that of a commercial sour beer in dried fruit odor, total flavor intensity, astringency, and acidic taste.

Another approach has been investigated in which cofermentation with *Lactobacillus paracasei* L26 and *S. cerevisiae* US-05 is used in sour beer production (114). A novel sour beer beverage with sufficiently high lactobacilli count to represent a legitimate delivery vehicle for probiotics was developed. Although the presence of ethanol in beer is

problematic in a health-promoting, probiotic context, the high viability of lactobacilli is noteworthy. The sour beer had a pH of 3.6 and contained 10^9 CFU of probiotic lactobacilli per serving (100 ml) and more than 5,000 mg/liter of lactic acid.

Conclusions. Interest in sour beer has increased substantially in recent decades. Sour beer is traditionally produced through spontaneous fermentations in which complex microbial consortia are involved (Fig. 3). These can include different yeast (*Saccharomyces* spp. and *Brettanomyces* spp.) and bacterial (*Lactobacillus* spp., *Pediococcus* spp., and *Acetobacter* spp.) species. A diverse range of metabolites are formed through the successive microbial progression of such fermentations, resulting in highly complex products with respect to sensory properties. High quantities of organic acids, such as lactic acid and acetic acid, results in low pH and high intensity in sourness and acidic taste compared to ales and lagers fermented by pure, single cultures of *S. cerevisiae* and *S. pastorianus*, respectively. Several issues complicate the production of sour beer through traditional methods. These include poor process control, lack of consistency in product quality, and lengthy fermentation times. Most of the sour beer research has been focused on understanding the complex spontaneous fermentation process, originating from traditional Belgian brewing culture. Pure-culture fermentations with strains of *Lactobacillus* and *S. cerevisiae*, in conjunction with careful application of processing steps, offer a valid alternative to facilitate production of sour beer with a higher level of process control and more rapid fermentation compared to traditional methods. Selection of strains based on their potential for substrate utilization and flavor generation could also open possibilities for using nonconventional sources of carbohydrates in beverages production through fermentation.

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